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The mitochondrial genome of the threatened freshwater snail *Aylacostoma chloroticum* (Gastropoda, Hemisinidae) from the High Paraná River and the phylogenetic relationships of Cerithioidea

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Abstract

The family Hemisinidae consists exclusively of freshwater snails and has long been understudied, only recently recognized as a distinct evolutionary lineage among limnic cerithioideans. Most South American species of this family belong to the genus *Aylacostoma*, with limited information available beyond their original descriptions. *Aylacostoma chloroticum*, a threatened freshwater snail endemic to the High Paraná River between Argentina and Paraguay, is currently part of an *ex-situ* conservation program due to habitat alterations caused by the Yacyretá Binational Reservoir. Molecular data on this species are scarce, with only one prior study addressing the genetic composition of captive populations. In this study, next-generation sequencing was used to sequence the complete mitochondrial genome of *A. chloroticum*, marking the first mitogenome contribution for both *Aylacostoma* and Hemisinidae. The mitogenome is 15,740 bp long and contains the typical 37 genes found in animal mitogenomes, including 13 protein-coding genes, 22 transfer RNAs, and two ribosomal RNAs. Comparative analyses revealed a conserved gene order, high A+T content, and negative AT and GC skews, consistent with other Cerithioidea species. Secondary structure models for ribosomal and transfer RNAs were also generated, providing the first complete models for *Aylacostoma* and Hemisinidae. Phylogenetic analyses, based on protein-coding genes and complementary analyses using *16S-rRNA* and *28S-rRNA* genes, confirmed the monophyly of Hemisinidae and its close evolutionary relationship with the families Paludomidae and Thiaridae. This research enhances the understanding of the mitochondrial architecture of Cerithioidea and provides new insights into the evolutionary relationships of Neotropical hemisinids.

Key Words

Mitochondrial DNA, molecular phylogenetics, Mollusca, secondary structure, South America

Introduction

Recent advances in DNA sequencing technologies have enabled the characterization of the mitochondrial genomes of numerous mollusk species, establishing this as one of the fastest-growing research areas in molluscan genomics (Gomes-dos-Santos et al. 2020; Ghiselli et al. 2021). Nonetheless, despite Mollusca being the second most species-rich animal phylum, the number of available mitogenomes remains remarkably low, representing less than 0.5% of the described species (Gomes-dos-Santos et al. 2020). In contrast to other animal groups, mitochondrial genomics in mollusks has revealed a high degree of heterogeneity in terms of genome length and architecture. This diversity includes significant size variation, relatively high rates of genomic rearrangements, gene

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duplication and loss, and a complex inheritance system (double uniparental inheritance), among other characteristics (Simison and Boore 2008; Kojima 2010; Osca et al. 2015; Ghiselli et al. 2021). Additionally, analyses based on complete mitochondrial genome sequences have been useful in exploring the evolutionary relationships within various molluscan groups, including octopuses, bivalves, and gastropods (e.g., Uribe and Zardoya 2017; Lee et al. 2019a; Guzmán et al. 2021).

Cerithioidea Fleming, 1822, is a vast superfamily of caenogastropods distributed worldwide, currently encompassing 22 families, 175 genera, and more than 1,500 described species, excluding fossil forms (MolluscaBase 2024). These species inhabit tropical, subtropical, and warm temperate regions, having successfully colonized marine, brackish, and freshwater environments (Strong et al. 2011). The last comprehensive phylogeny of the Cerithioidea, undertaken by Strong et al. (2011), used both morphological and molecular data from representatives across 17 families, revealing that colonization of continental waters occurred through two or three independent lineages. Within the superfamily, Hemisinidae Fischer & Crosse, 1891 is unique to the Neotropics and comprises exclusively freshwater snails, representing a significant component of the freshwater malacofauna (Glaubrecht and Neiber 2019). The shells of Hemisinidae range from medium to large and are generally elongated-oval to oval-conical, often adorned with spiral lines and occasionally marked by sinuous ribs or nodules at the shoulders of the whorls (Glaubrecht and Neiber 2019). Previously classified as part of the familiy Thiaridae Gill, 1871 (1823), Hemisinidae has been historically understudied and only recently recognized as a distinct evolutionary lineage among limnic cerithioideans (Strong et al. 2011; Gimnich 2015; Glaubrecht and Neiber 2019).

While several genus-group names exist for Neotropical hemisinids, such as *Aylacostoma* Spix, 1827, *Hemisinus* Swainson, 1840, *Verena* Adams & Adams, 1854, *Longiverena* Pilsbry & Olsson, 1935, and *Basistoma* Lea, 1852 (Morrison 1954; Nuttall 1990; Glaubrecht and Neiber 2019), their taxonomic boundaries have yet to be clearly defined. Most of the South American representatives of Hemisinidae are classified under the genus *Aylacostoma*, which encompasses over 30 species (Morrison 1954; Simone 2006; MolluscaBase 2024). Despite their diversity, information on these species is predominantly restricted to their original descriptions (Vogler et al. 2014). Species descriptions within this genus for Argentina and Paraguay were first conducted by Hylton Scott (1953, 1954) in the High Paraná River region, describing *Aylacostoma guaraniticum* (Hylton Scott, 1953), *A. stigmaticum* Hylton Scott, 1954, and *A. chloroticum* Hylton Scott, 1954. These species formerly inhabited the Yacyretá-Apipé rapids in the Paraná River, an area now submerged by the Yacyretá Binational Reservoir (Vogler et al. 2016). The first two species have become extinct due to habitat alterations caused by the filling of the reservoir (Peso et al. 2013a, 2013b; Vogler 2013). However, various populations of *A. chloroticum* are being bred in captivity through the "Proyecto *Aylacostoma*", an *ex-situ* conservation initiative that has been active since the 1990s and involves multiple institutions (Miyahira et al. 2022; Peso 2022). Despite this, the available information for the species is very limited, with some ecological data provided in the early 2000s (Quintana et al. 2001) and the first anatomical descriptions only in 2014 (Vogler et al. 2014). At the molecular level, knowledge of *A. chloroticum* is even more scarce. To date, only one phylogeographic study has been developed with a conservation focus to shed light on the genetic composition of captive populations and their past history. This study was restricted to a single mitochondrial marker, the cytochrome *c* oxidase subunit I (*cox1*) gene, and documented very low genetic diversity (Vogler et al. 2015). Furthermore, within the framework of a historical DNA study on the extinct *A. stigmaticum*, the first partial mitochondrial *12S-rRNA* sequences for a few *A. chloroticum* specimens were obtained from a limited dataset, which included representatives from two captive-bred populations and were characterized by a single haplotype (Vogler et al. 2016). Moreover, to our knowledge, the evolutionary affinities of *Aylacostoma* or any other South American member of Hemisinidae within an evolutionary systematic context of Cerithioidea, based on molecular data, have never been explored. Some morphological studies, however, have indicated that the group shares anatomical characteristics with the families Thiaridae and Paludomidae (Simone 2001; Gomez et al. 2011; Glaubrecht and Neiber 2019).

Recent advances have improved our understanding of the mitochondrial genomes of various members of the Cerithioidea superfamily, particularly in terms of their phylogenetic relationships based on complete mitogenome data (e.g., Kato et al. 2022; Ling et al. 2022; Yang and Deng 2022; Yin et al. 2022; Xu et al. 2024). Moreover, two distinct genetic rearrangements have been identified within the superfamily, especially concerning the positioning of tRNAs for Arginine and Glutamine (Kato et al. 2022). Despite these advancements, the availability of sequences remains limited. The GenBank database currently lists 439 reference mitogenomes for Gastropoda, of which only 11 belong to Cerithioidea, spanning six families. Notably, there are still no representatives from the South American Hemisinidae (NCBI 2024).

Given the above, and considering the limited molecular information available from any South American hemisinid, several key questions arise: Does *Aylacostoma* truly belong to a distinct evolutionary lineage currently recognized as Hemisinidae, or does it represent a member of Thiaridae as historically proposed? Furthermore, since alternative gene arrangements have been characterized within the mitogenomes of Cerithioidea, could *Aylacostoma* exhibit a different gene order compared to other members of the superfamily? Do its structural and

compositional characteristics align with those known for other members of the group, or are they significantly different? Lastly, are the phylogenetic relationships based on complete mitogenomes of Cerithioidea consistent with those derived from single genes?

To address these questions, we sequenced and characterized the mitochondrial genome of the threatened *Aylacostoma chloroticum*, providing fundamental information about its mitochondrial features. Specifically, the study aimed to investigate (i) the composition, organization, and structural characteristics of the assembled mitogenome, comparing it with published data for Cerithioidea to evaluate similarities and differences, and (ii) to explore the phylogenetic relationships of the genus *Aylacostoma* and the Hemisinidae family within the Cerithioidea context, establishing its evolutionary affinities based on complete mitogenome data in contrast to those from single genes. Considering that limited anatomical information on Hemisinidae suggests an affinity with the Thiaridae and Paludomidae families, we expect to find similar structural and compositional characteristics among the mitogenomes of these families. Based on existing knowledge, we anticipate finding similarities in AT content, AT and GC skew values, sizes of the protein-coding genes, and codon usage, among other aspects. We also hypothesize that they share the same type of gene arrangement. Furthermore, we hypothesize that phylogenetic reconstructions based on both complete mitogenomes and single genes will reveal close evolutionary relationships among these families, potentially reinforcing the proposed Gondwanan origin of this group.

Materials and methods

Sample, DNA extraction and sequencing

This study was based on a specimen of *A. chloroticum* housed in the Malacological Collection of the Instituto de Biología Subtropical (IBS-Ma 1082-11; CONICET–Universidad Nacional de Misiones, Misiones, Argentina). The specimen, collected from the High Paraná River at Candelaria, Misiones, Argentina (27.447488°S, 55.750233°W), was integrated into the *ex-situ* conservation program managed by the Universidad Nacional de Misiones (Posadas, Argentina). It was identified based on conchological features and by analyzing partial sequences of the *cox1* gene following Vogler et al. (2014). Total genomic DNA was extracted from a section of muscular foot tissue, preserved at -20 °C in 0.9% saline solution (NaCl), using a cetyltrimethylammonium bromide (CTAB) protocol (Beltramino et al. 2018). The mitochondrial genome was sequenced by Novogene Corporation (Sacramento, CA, USA) employing next-generation sequencing (NGS) technology, specifically through paired-end sequencing (2 × 150 base pairs, bp) with an insert size of 350 bp on an Illumina HiSeq platform.

Mitogenome assembly and annotation

The assembly of the mitochondrial genome of *A. chloroticum* was performed automatically using NOVOPlasty 4.3.1 (Dierckxsens et al. 2017), with the partial sequence of the *cox1* gene serving as the seed. For the mitogenome annotation, we utilized the MITOS, MITOS 2 and GeSeq web servers (Bernt et al. 2013a; Tillich et al. 2017), along with MitoZ 3.5 and MitoFinder 1.4.2 programs (Meng et al. 2019; Allio et al. 2020), employing the mitochondrial genetic code for invertebrates. The NCBI ORF Finder resource (https://www.ncbi.nlm.nih.gov/orffinder/) was also used to identify and characterize the protein-coding genes (PCGs). Manual adjustments were made to ensure the accuracy of the start and stop codons and the boundaries of genes, supported by BLASTp comparisons (Altschul et al. 1997) against known protein sequences. The transfer RNA genes (tRNAs) were also identified using the ARWEN 1.2 web server (Laslett and Canbäck 2008), whereas ribosomal RNA genes (rRNAs) were annotated through comparative analyses with other molluscan mitogenomes, with the boundaries of the rRNAs manually adjusted according to the locations of adjacent genes (Boore et al. 2005; Cameron 2014). Additionally, for subsequent comparison, the annotation of some Cerithioidea mitogenomes was refined using the tools implemented for *A. chloroticum* (Suppl. material 1).

Following annotation, the nucleotide composition and relative synonymous codon usage (RSCU) for PCGs were analyzed using MEGA 11 software (Tamura et al. 2021). Global AT and GC skews were calculated using the equations of Perna and Kocher (1995): AT skew = (A-T) / (A+T) and GC skew = (G-C) / (G+C). Subsequently, skew values were further assessed through a sliding windows analysis with partial overlap using the routines StrandCG and StrandAT (Ghiringhelli, unpublished), with a window size of 100 bp and a step size of 30 bp. To provide a visual representation, the mitochondrial genome was depicted in a circular format using the online server Proksee (Grant et al. 2023).

Secondary structure models of tRNAs and rRNAs

Structural models for tRNAs were initially generated using ARWEN 1.2 and subsequently refined through manual adjustments based on Boore et al. (2005). For the ribosomal genes (*12S-rRNA* and *16S-rRNA*), secondary structure models were first derived using the R2DT web server (Sweeney et al. 2021), supported by the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). These initial models were further refined through manual corrections. The refinement process involved utilizing several reference models specific to mollusks (Lydeard et al. 2000; Guzmán et al. 2021; Mendivil et al. 2023), as well as other eukaryotic models available from the Comparative RNA Web-2 site (https://crw2-comparative-rna-web.org/).

Phylogenetic analyses

Phylogenetic trees were constructed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods based on sequences of the 13 PCGs from *A. chloroticum* and 18 other Cerithioidea species, with two additional caenogastropods serving as outgroups (Suppl. material 2). The mitogenomes of the cerithioideans *Batillaria cumingii* (Crosse, 1862) (MT323103), *Batillaria zonalis* (Bruguière, 1792) (MT363252), and *Paludomus ajanensis* Morelet, 1860 (PP946412) were excluded from our analyses due to the lack of annotations in GenBank and the absence of annotations from the original sources (Yan et al. 2020a, 2020b; Stelbrink et al. 2024). Additionally, the mitogenome of *Koreoleptoxis globus* (Martens, 1886) (LC006055, unpublished), annotated on the complementary strand in GenBank, was also omitted. Analyses comprised three datasets: one based on amino acid sequences (aa dataset) and two based on nucleotide sequences, the PCG123 dataset, which included all three codon positions, and the PCG12 dataset, which incorporated only the first two codon positions.

The analysis workflow was conducted using PhyloSuite 1.2.3 (Zhang et al. 2020; Xiang et al. 2023), which included the following steps: i- alignment of sequences using MAFFT 7.505 (Katoh and Standley 2013); ii- refinement of nucleotide sequence alignments with MACSE 2.06 (Ranwez et al. 2018); iii- removal of ambiguous sites using Gblocks 0.91b (Talavera and Castresana 2007) in codon mode for the PCGs and trimAI 1.2 (Capella-Gutiérrez et al. 2009) for the aa dataset; iv- concatenation of all 13 PCGs; and v- model selection using ModelFinder 2.2.0 (Kalyaanamoorthy et al. 2017), with selection based on the Bayesian Information Criterion (BIC; Suppl. material 3). The final datasets comprised 3,720 amino acids for the amino acid matrix and 11,205 and 7,470 nucleotides for the PCG123 and PCG12 matrices, respectively. ML analyses were conducted using IQ-TREE 2.2.0 (Minh et al. 2020) with 10,000 ultrafast bootstrap replicates (Hoang et al. 2018). BI analyses were performed using MrBayes 3.2.7a (Ronquist et al. 2012), based on two independent runs with four Markov chains running simultaneously for 10^6 generations, sampling every 100 generations, with a final burn-in of 25%. The convergence of independent runs was assessed by examining the mean standard deviation of split frequencies (< 0.01). BI analyses were implemented in MrBayes 3.2.7a on the CIPRES Science Gateway platform (Miller et al. 2010). The resultant phylogenetic trees were visualized using the iTOL 6 web server (Letunic and Bork 2024).

In addition to mitogenomic-based approaches, phylogenetic reconstructions using the *16S-rRNA* and *28S-rRNA* markers were also performed to assess the evolutionary relationships of *A. chloroticum* within the Cerithioidea superfamily. This aligns with the comprehensive phylogenetic framework proposed by Strong et al. (2011) which utilized single-gene data. The phylogenetic analyses included sequences of the *16S-rRNA* and *28S-rRNA* genes from *A. chloroticum* and 60 other Cerithioidea species, with nine additional species as outgroups (Suppl. material 4). The *28S-rRNA* sequence for *A. chloroticum* was obtained from the genomic data using Novoplasty 4.3.1, with a sequence from *Tarebia granifera* (Lamarck, 1816) (HM003668) serving as the seed. The phylogenetic workflow, executed in PhyloSuite 1.2.3, mirrored previous descriptions, involving removal of ambiguous sites through trimAI, but without the alignment refinement step with MACSE 2.06. The concatenated dataset comprised 1,751 nucleotides. Phylogenetic trees were constructed using both BI and ML methods, as previously described for the datasets based on mitogenomes. ModelFinder 2.2.0 was used to select the most appropriate substitution models based on the BIC, identifying GTR+F+I+G4 as suitable for both markers in BI analysis, and GTR+F+I+I+R4 and TN+F+R3 for the *16S-rRNA* and *28S-rRNA*, respectively, in ML analysis.

Results

Structure and nucleotide composition of the mitogenome

The mitochondrial genome of *Aylacostoma chloroticum* (Accession Number: PQ279514) spanned 15,740 bp and included the typical set of 37 genes—13 PCGs, 2 rRNAs and 22 tRNAs (Fig. 1, Table 1). Additionally, 21 intergenic regions ranging from 1 to 511 bp were identified, totaling 4.67% of the genome (735 bp). A significant non-coding region, 511 bp in length, was located between the genes for $tRNA^{Phe}$ and $tRNA^{Cys}$ (Table 1). The mitogenome also included 4 overlapping regions, with the longest being 47 bp between the *cob* and *nad6* genes (Table 1). The nucleotide composition of the mitogenome was determined as 32.44% A, 35.03% T, 17.31% C and 15.22% G, demonstrating a high AT content of 67.47%. The AT and GC skew values were negative, calculated at -0.0384 and -0.0641, respectively (Suppl. material 1). The AT and GC skews computed using a sliding window strategy with partial overlap are shown in Fig. 2.

Protein-coding genes and codon usage

The PCGs accounted for approximately 71.80% of the mitogenome, totaling 11,301 bp. Of these, nine were encoded on the plus strand, and four on the minus strand. All PCGs started with the ATG start codon, while the stop codons varied between TAA and TAG (Table 1). Excluding start and stop codons, a total of 3,741 codons were identified. The relative synonymous codon usage pattern is presented in Fig. 3 and Suppl. material 5.

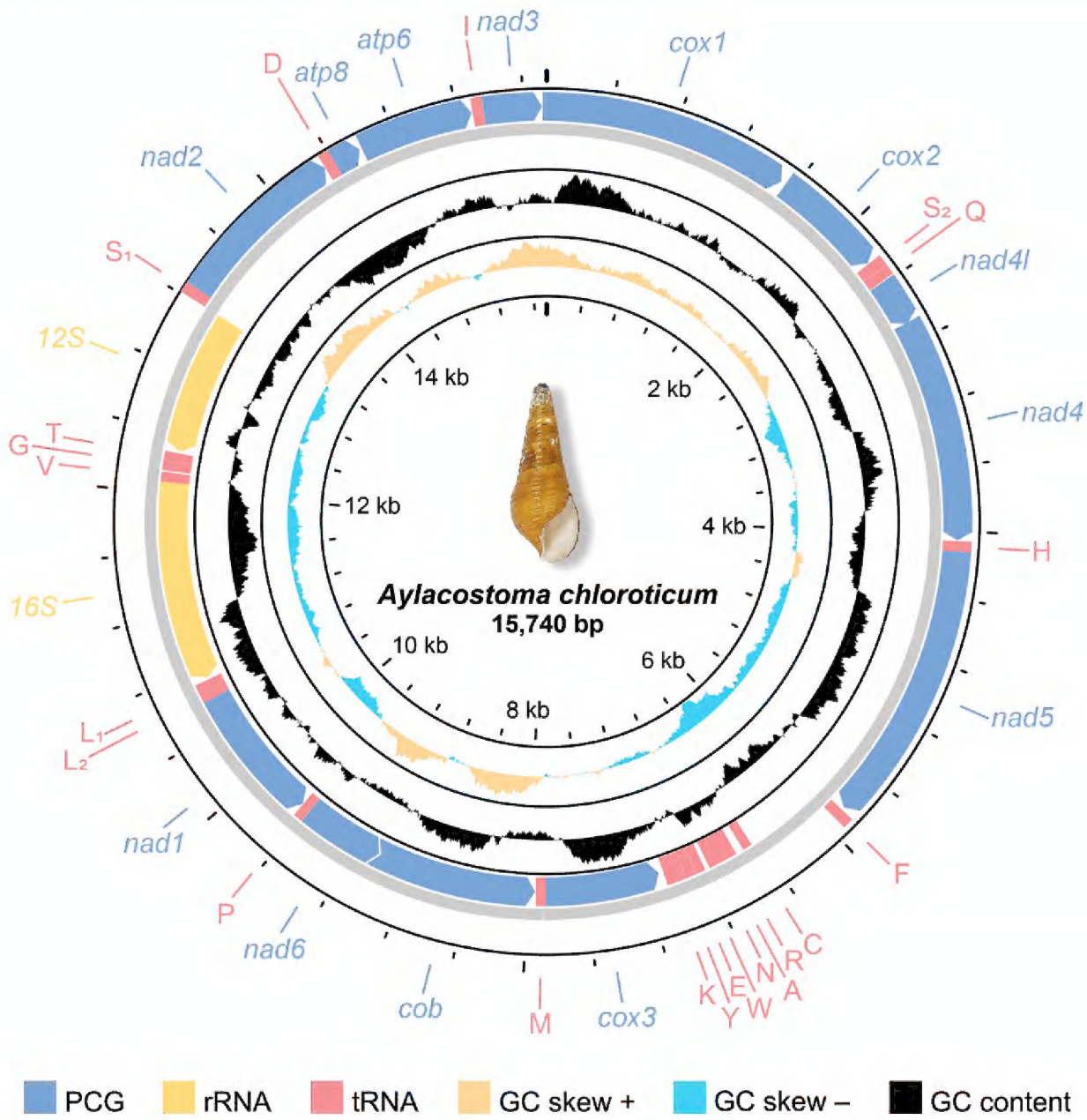

Figure 1. Mitochondrial genome of *Aylacostoma chloroticum*. From the center outward, the diagram shows the genome size, GC skew, GC content, and gene organization. Genes encoded on the plus strand are illustrated outside the circle, while those on the minus strand are inside. The three types of genes are differentiated by color as referenced in the figure. PCG: protein-coding gene. The tRNAs are presented with one-letter abbreviation codes.

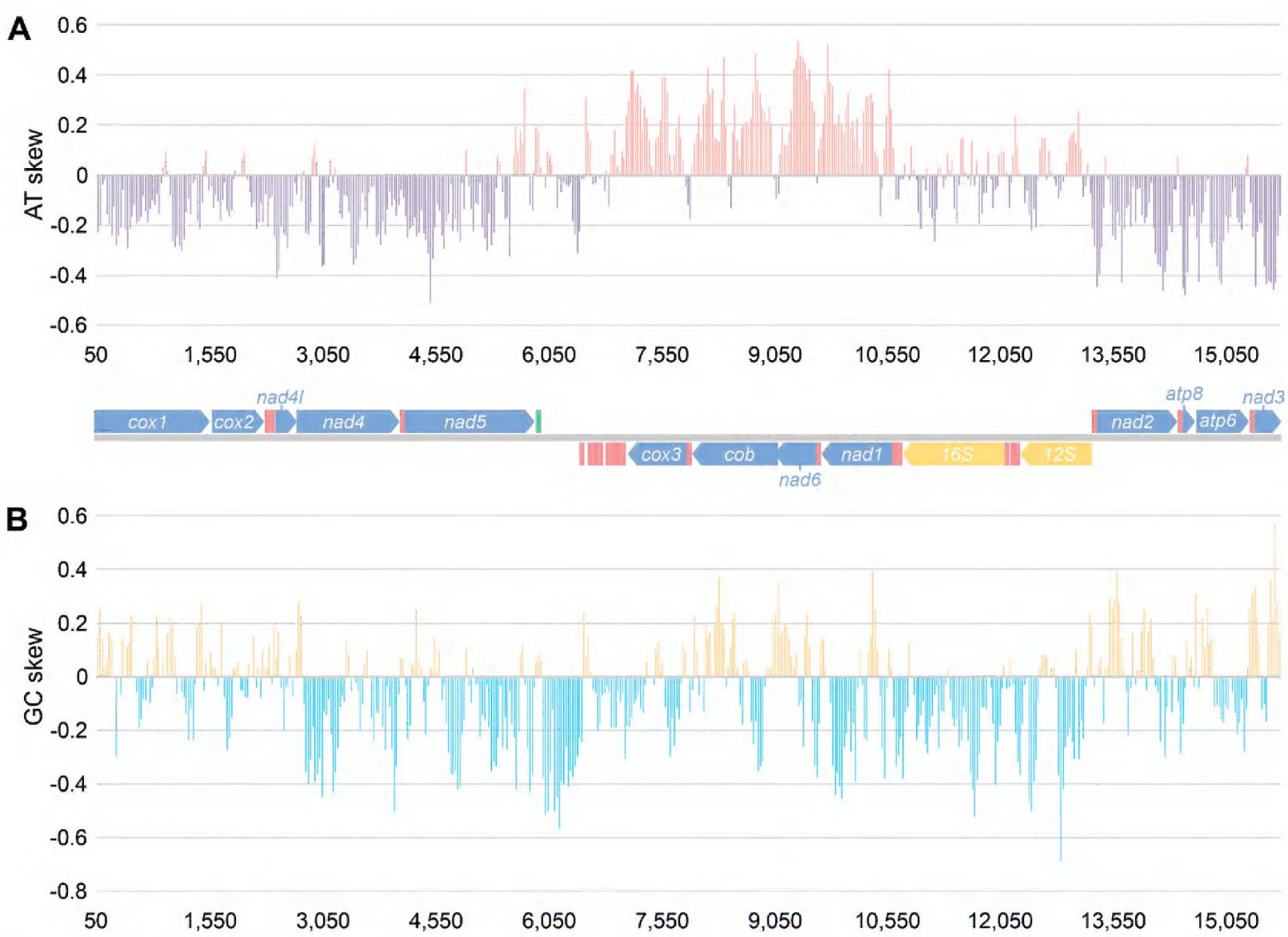

Figure 2. AT and GC skews using a sliding window strategy for the mitogenome of *Aylacostoma chloroticum*. **A.** AT skew; **B.** GC skew.

Table 1. Organization of the mitochondrial genome of *Aylacostoma chloroticum*.

Gene	Strand	From	To	Size (bp)	Start codon	Stop codon	Anticodon	Intergenic Nucleotides
cox1	+	1	1,533	1,533	ATG	TAA		+30
cox2	+	1,564	2,253	690	ATG	TAA		+6
tRNA^{Ser2}	+	2,260	2,326	67			TGA	+1
tRNA^{Gln}	+	2,328	2,393	66			TTG	+5
nad4l	+	2,399	2,689	291	ATG	TAA		-7
nad4	+	2,683	4,050	1,368	ATG	TAA		+3
tRNA^{His}	+	4,054	4,118	65			GTG	0
nad5	+	4,119	5,837	1,719	ATG	TAA		+18
tRNA^{Phe}	+	5,856	5,920	65			GAA	+511
tRNA^{Cys}	–	6,432	6,495	64			GCA	+53
tRNA^{Arg}	–	6,549	6,614	66			TCG	0
tRNA^{Ala}	–	6,615	6,679	65			TGC	-2
tRNA^{Asn}	–	6,678	6,745	68			GTT	+30
tRNA^{Trp}	–	6,776	6,842	67			TCA	+1
tRNA^{Glu}	–	6,844	6,909	66			TTC	+1
tRNA^{Tyr}	–	6,911	6,976	66			GTA	-2
tRNA^{Lys}	–	6,975	7,040	66			TTT	+31
cox3	–	7,072	7,851	780	ATG	TAA		0
tRNA^{Met}	–	7,852	7,918	67			CAT	+5
cob	–	7,924	9,063	1,140	ATG	TAA		-47
nad6	–	9,017	9,568	552	ATG	TAA		+1
tRNA^{Pro}	–	9,570	9,633	64			TGG	+3
nad1	–	9,637	10,575	939	ATG	TAG		0
tRNA^{Leu2}	–	10,576	10,641	66			TAA	0
tRNA^{Leu1}	–	10,642	10,709	68			TAG	0
16S-rRNA	–	10,710	12,066	1,357				0
tRNA^{Val}	–	12,067	12,133	67			TAC	+9
tRNA^{Gly}	–	12,143	12,208	66			TCC	0
tRNA^{Thr}	–	12,209	12,276	68			TGT	0
12S-rRNA	–	12,277	13,224	948				0
tRNA^{Ser1}	+	13,225	13,291	67			GCT	0
nad2	+	13,292	14,359	1,068	ATG	TAA		+1
tRNA^{Asp}	+	14,361	14,426	66			GTC	0
atp8	+	14,427	14,594	168	ATG	TAG		+15
atp6	+	14,610	15,308	699	ATG	TAA		+6
tRNA^{Ile}	+	15,315	15,381	67			GAT	+1
nad3	+	15,383	15,736	354	ATG	TAG		+4

Values with a minus sign in intergenic nucleotides indicate overlapping sequences between adjacent genes.

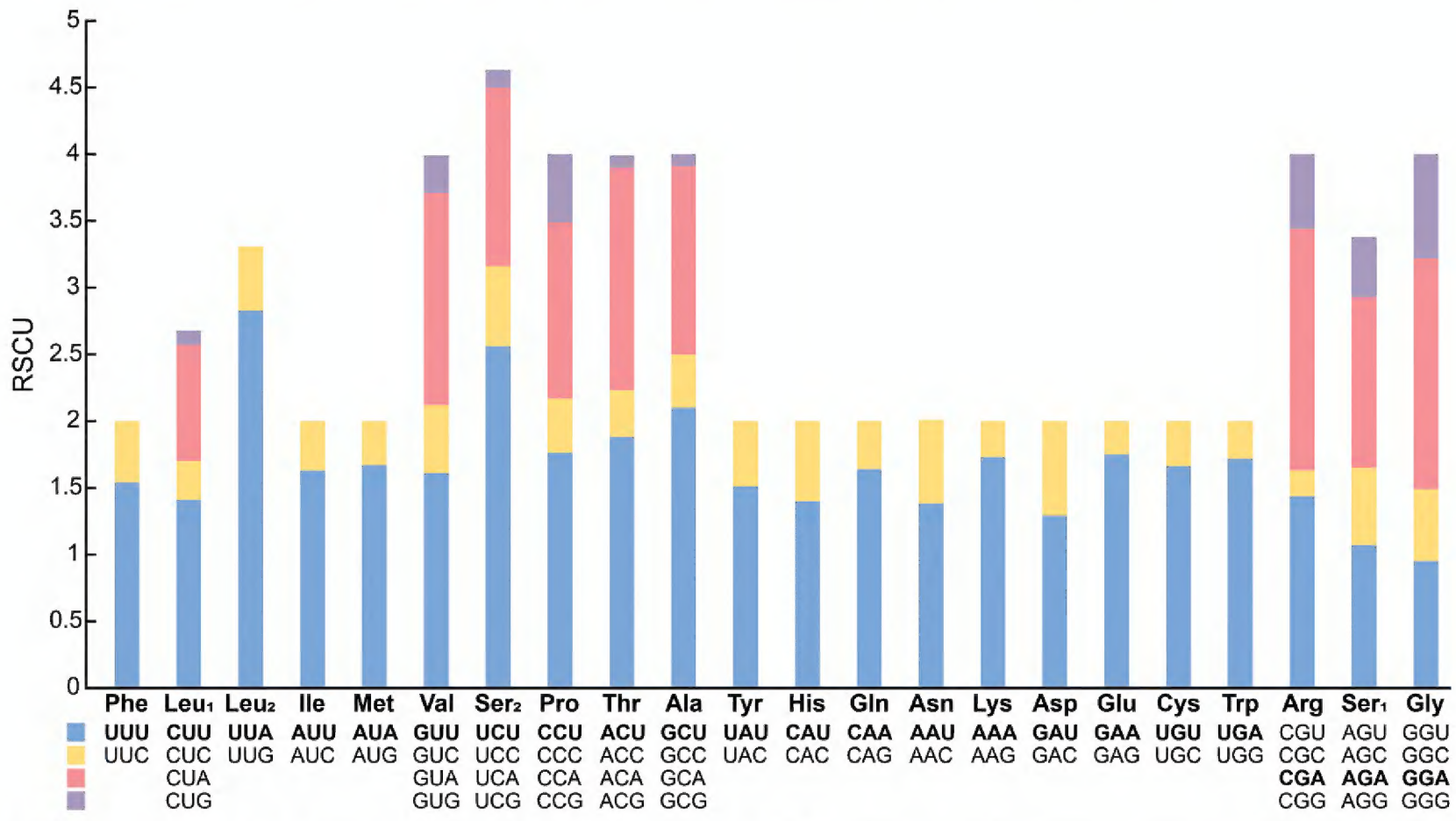
RSCU
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Phe Leu1 Leu2 Ile Met Val Ser2 Pro Thr Ala Tyr His Gln Asn Lys Asp Glu Cys Trp Arg Ser1 Gly
UUU CUU UUA AUU AUA GUU UCU CCU ACU GCU UAU CAU CAA AAU AAA GAU GAA UGU UGA CGU AGU GGU
UUC CUC UUG AUC AUG GUC UCC CCC ACC GCC UAC CAC CAG AAC AAG GAC GAG UGC UGG CGC AGC GGC
CUA GUA UCA CCA ACA GCA CGA AGA GGA
CUG GUG UCG CCG ACG GCG CGG AGG GGG

Figure 3. Relative Synonymous Codon Usage (RSCU) of protein-coding genes in the mitogenome of *Aylacostoma chloroticum*. The most frequently used codons are highlighted in bold.

Ribosomal and transfer RNA genes

The 22 tRNAs comprised 9.26% of the mitogenome, totaling 1,457 bp, with seven encoded on the plus strand and 15 on the minus strand (Fig. 1, Table 1). The tRNAs varied in size from 64 to 68 nucleotides (Table 1). Most adopted the typical cloverleaf secondary structure, featuring an acceptor stem, TΨC and DHU arms, a variable arm, and an anticodon stem and loop; however, tRNA^{Ser1} deviated by featuring a large loop instead of the typical stem-loop structure in the DHU arm. The proposed secondary structures for the tRNAs of *A. chloroticum* are presented in Suppl. material 6: fig. S1. Additionally, the rRNA genes, which account for approximately 14.64% of the mitogenome, were both encoded on the minus strand (Fig. 1). The *16S-rRNA*, located between the tRNA^{Leu1} and tRNA^{Val} genes, was 1,357 bp long (Table 1), with its secondary structure shown in Suppl. material 6: figs S2, S3. The *12S-rRNA*, spanning 948 bp, was situated between the tRNA^{Thr} and tRNA^{Ser1} genes (Fig. 1, Table 1), with its secondary structure model illustrated in Suppl. material 6: fig. S4.

Phylogenetic analyses and gene organization

The results of phylogenetic reconstructions using BI and ML based on mitogenomes were identical in topology across the implemented strategies for the PCG123 and PCG12 datasets, with the exception observed in the trees obtained based on the aa dataset. Here, the topologies were consistent between both methods but differed from the nucleotide-based in terms of the affinities of the family Potamididae (Fig. 4). In all cases, the superfamily Cerithioidea was recovered as a monophyletic group with maximum support values. In the nucleotide-based topologies, Hemisinidae grouped as a sister clade to Thiaridae, with Paludomidae positioned externally to both families, and Potamididae being the sister group to these. In contrast, in the topology based on amino acids, Potamididae related to the group formed by Semisulcospiridae + Pleuroceridae, albeit with low support values. The comparison of gene organization in the mitogenomes of different families within the Cerithioidea superfamily is presented in Fig. 4C. Phylogenetic reconstructions from the concatenated analysis of the *16S-rRNA* and *28S-rRNA* genes under BI and ML are shown in Fig. 5. The obtained topologies were similar, although there were slight differences in the internal organization of groups. For both methods, the Hemisinidae family was recovered as monophyletic, with maximum support values, grouping *A. chloroticum* together with *Hemisinus cubanianus* (d'Orbigny, 1842).

Discussion

We successfully sequenced and characterized the mitochondrial genome of the threatened South American gastropod *Aylacostoma chloroticum*, which spans 15,740 bp. To our knowledge, this represents the first complete mitochondrial genome reported for any species within the genus *Aylacostoma* and the family Hemisinidae. The length of the mitogenome falls within the range previously reported for Cerithioidea (Suppl. material 1),

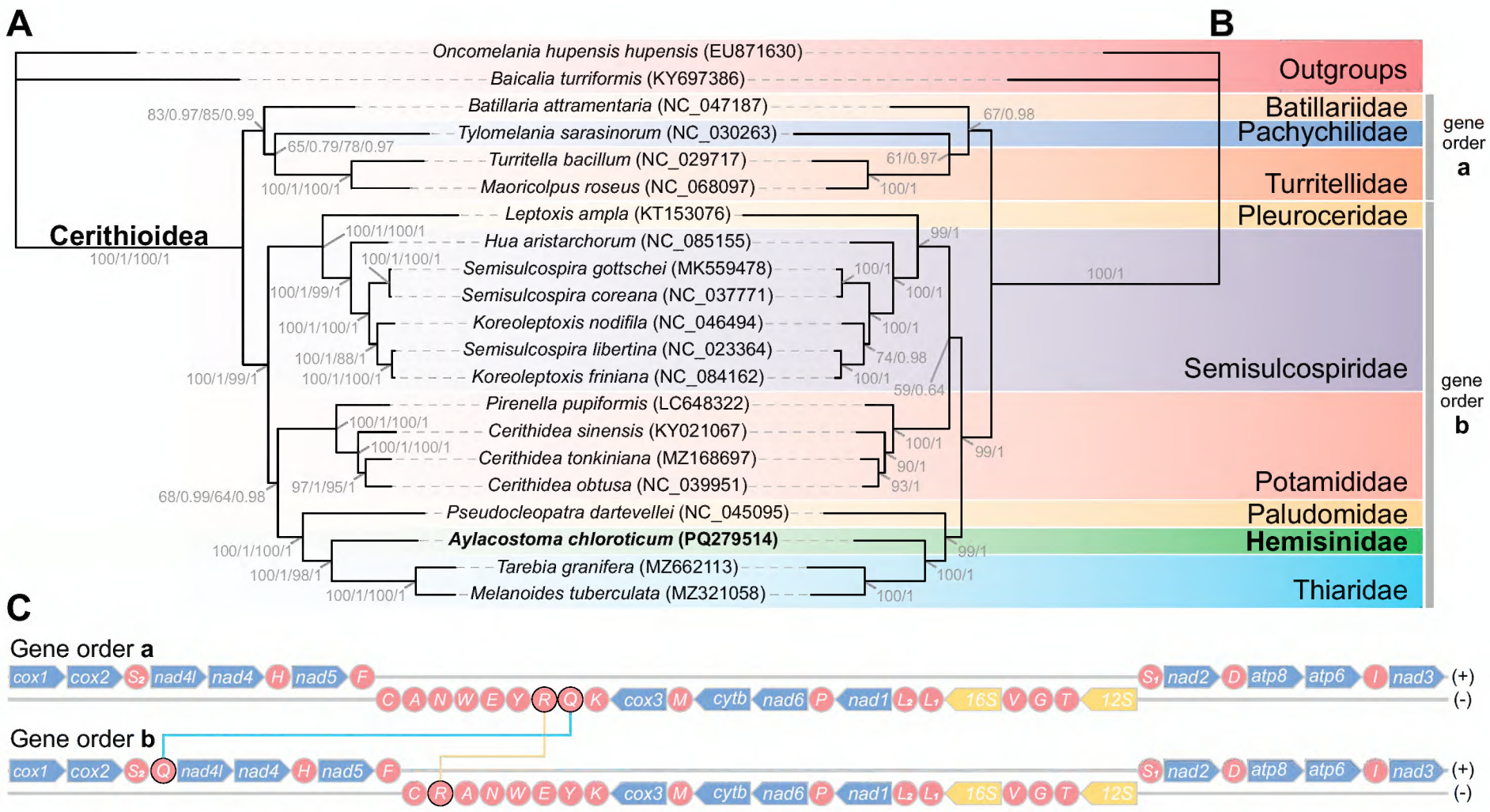

Figure 4. Phylogenetic relationships of Cerithioidea based on complete mitogenomes using 13 protein-coding genes and gene order. **A.** ML tree based on nucleotide sequences; bootstrap support values and posterior probabilities (> 50/0.5) for the PCG123 and PCG12 datasets are shown in the following order: PCG123 (ML) / PCG123 (BI) / PCG12 (ML) / PCG12 (BI); **B.** ML tree based on amino acid sequences; bootstrap support values and posterior probabilities (> 50/0.5) are shown; **C.** Gene arrangements within Cerithioidea.

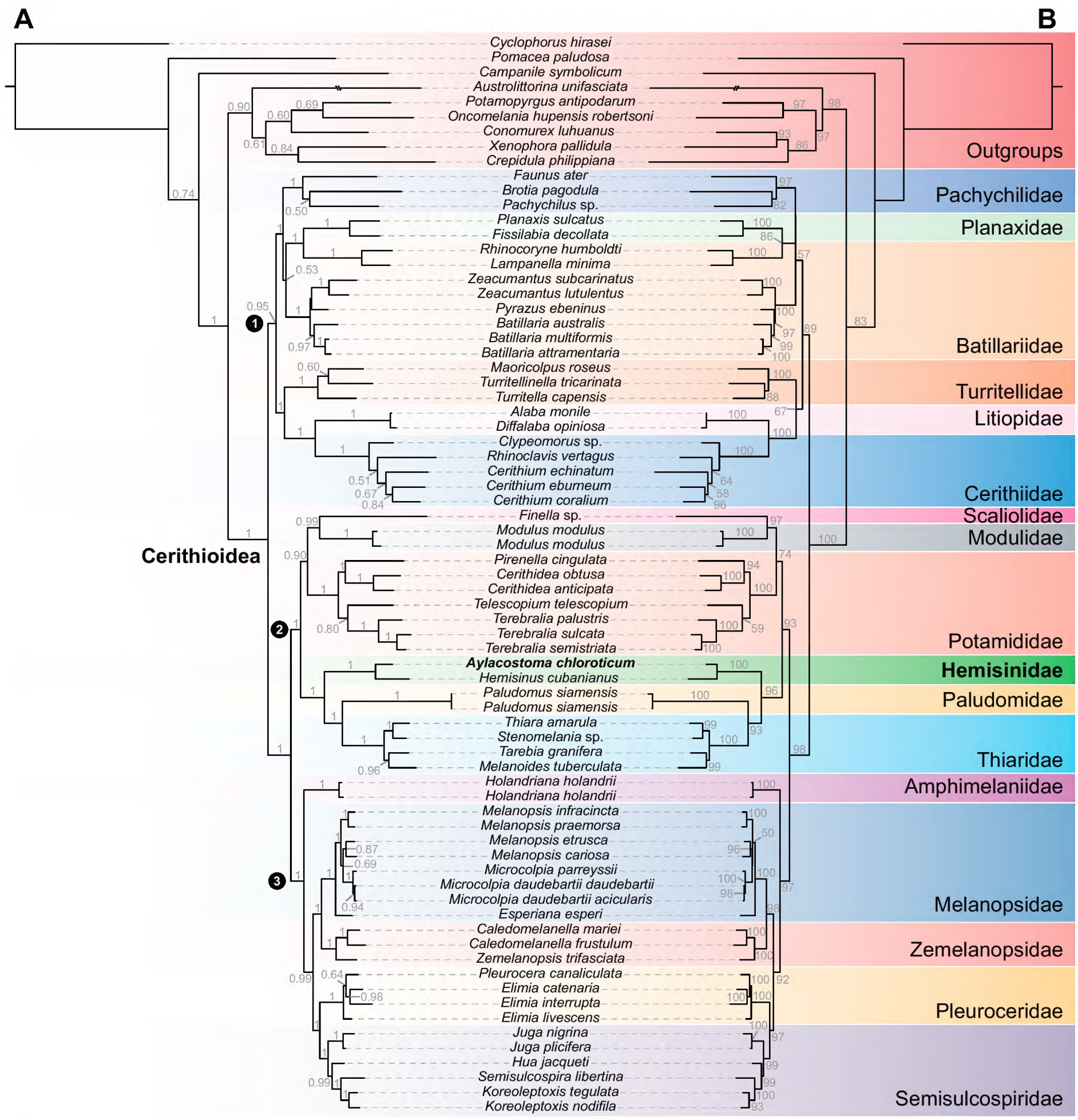

Figure 5. Phylogenetic trees of Cerithioidea based on the combined analysis of *16S-rRNA* and *28S-rRNA* markers. **A.** BI tree; **B.** ML tree. Bootstrap support values for ML and posterior probabilities for BI are indicated (> 50/0.5). Numbers in a black circle represent the three main assemblages of cerithioidean as defined by Strong et al. (2011).

extending from 15,368 bp in the paludomid *Pseudocleopatra dartevellei* Mandahl-Barth, 1973 from the Congo River, Democratic Republic of the Congo (Stelbrink et al. 2019), to 16,632 bp in the pachychilid *Tylomelania sarasinorum* (Kruimel, 1913) from Sulawesi, Indonesia (Hilgers et al. 2016). This variation in size is primarily attributed to differences in the lengths of the non-coding regions (Lee et al. 2019b).

The mitogenome of *A. chloroticum* contains the 37 genes typical of Metazoa, including 13 PCGs, along with two rRNA genes and 22 tRNA genes. Of these, 16 are encoded on the plus strand (+) and 21 on the minus strand (–), with the designation of the plus strand primarily based on the orientation of the *cox1* gene—an arbitrary convention, given the variability in gene order and relative orientation (Bernt et al. 2013b). Although the encoding of most genes on the minus strand appears to be the common pattern within Cerithioidea (Suppl. material 1; Lee et al. 2019b), the reverse pattern is observed in other superfamilies within the subclass Caenogastropoda, where a greater number of genes are typically encoded on the plus strand (Arquez et al. 2012; Osca et al. 2015). Furthermore, it is essential to distinguish this nomenclature from the definitions of major and minor coding strands, which are based on the number of genes encoded, as well as from the heavy (H) and light (L) strands, which are

based on G+T content, with the G+T-rich strand designated as the heavy strand (Bernt et al. 2013b; Barroso Lima and Prosdocimi 2018; Sun et al. 2018). Consistent with this, our examination of the 19 Cerithioidea mitogenomes, including that of *A. chloroticum*, confirms that the majority of genes are encoded on the light strand (which is also the major strand). However, the application of this nomenclature in the literature concerning Cerithioidea mitogenomes has proven inconsistent. For instance, genes are accurately assigned to heavy and light strands for the semisulcospirid *Semisulcospira coreana* (Martens, 1886), while for the turritellid *Turritella bacillum* Kiener, 1843, strand assignments are inaccurately reported (Zeng et al. 2016; Kim and Lee 2018). Such inconsistencies are prevalent across animal mitogenomes and represent a common source of confusion regarding strand nomenclature for mitochondrial DNA (Barroso Lima and Prosdocimi 2018; Shokolenko and Alexeyev 2022).

In relation to nucleotide composition, the mitogenome of *A. chloroticum* exhibits a high A+T content of 67.47%. This value falls within the reported range for Cerithioidea and represents the second highest among the characterized mitogenomes for the superfamily to date (Suppl. material 1), varying from 62.91% in the Southeast Asian potamidid *Cerithidea obtusa* (Lamarck, 1822) to 68.82% in the North American pleurocerid *Leptoxis ampla* (Anthony, 1855) (Whelan and Strong 2016; Nguyen et al. 2018). This observation aligns with the broader trend that mitochondrial genomes in mollusks are typically characterized by a strong compositional bias, particularly rich in A and T nucleotides (Sun et al. 2018). In the mitogenome of *A. chloroticum*, we also recorded four overlapping regions, two of which occurred between tRNAs and two between PCGs (Table 1). The longest overlapping region, extending 47 bp, was found between the *cob* and *nad6* genes, a feature previously reported in other Cerithioidea species (e.g., Hilgers et al. 2016; Lee et al. 2019b; Stelbrink et al. 2019). Additionally, an intergenic region of 511 bp, rich in A+T (A+T content = 70.7%), was found between the $tRNA^{Phe}$ and $tRNA^{Cys}$ genes. An examination of Cerithioidea mitogenomes deposited in GenBank indicates that the intergenic region between those genes is variable in size and has been annotated as the "control region" in several species, such as the thiarid *Melanoides tuberculata* (Müller, 1774) (MZ321058), the turritellid *T. bacillum* (NC029717), and the potamidid *Cerithidea tonkiniana* Mabille, 1887 (MZ168697), without providing additional characterization or detailed information (Zeng et al. 2016; Ling et al. 2022; Yang and Deng 2022). Therefore, it is speculated that this region could correspond to the putative control region in *A. chloroticum*. Nonetheless, since the control region of Cerithioidea remains poorly characterized, complementary studies aimed at identifying the structural elements involved in the regulation of transcription and replication found in other mollusk species (Ghiselli et al. 2021, and references therein) are needed to definitively designate this large intergenic region as the control region in *A. chloroticum*.

Regarding strand asymmetry analysis, the mitogenome of *A. chloroticum* exhibited global negative values for both AT and GC skews (-0.0384 and -0.0641, respectively), with the plus strand showing a bias toward T and C at the expense of A and G. In metazoans, two distinct clusters have been characterized based on global strand asymmetry: the first, exhibiting positive AT and negative GC skews, while the second is characterized by reversed strand asymmetry, displaying inverse values (Bernt et al. 2013b; Sun et al. 2018). Among mollusks, cephalopods and a few gastropod species belong to the first group, whereas bivalves and most gastropod species exhibit reversed strand asymmetry (Sun et al. 2018). In this study, out of the 19 Cerithioidea species, six conformed to the reversed pattern of negative AT and positive GC skews [the potamidids *Pirenella pupiformis* Ozawa & Reid, 2016, *Cerithidea sinensis* (Philippi, 1848), the paludomid *P. dartevellei*, the pachychilid *T. sarasinorum*, and the turritellids *T. bacillum* and *Maoricolpus roseus* (Quoy & Gaimard, 1834)], whereas the remaining species displayed negative values for both skews (Suppl. material 1). To our knowledge, studies focused on understanding the mutation processes that shape strand asymmetry are nonexistent for Cerithioidea. However, in other animal groups, it has been found that the relative contributions of different substitution types to strand asymmetry are associated with either replication alone or both replication and transcription (Wei et al. 2010; Fonseca et al. 2014). During these processes, one strand is transiently in a single-stranded state, making it more susceptible to DNA damage, which has been widely considered to influence the mutation bias between the two complementary DNA strands (Hassanin et al. 2005; Sun et al. 2018). Additionally, the sliding window analysis revealed a reversal in AT skew for *A. chloroticum*, which appears to be associated with the orientation of PCGs in the DNA strands. As far as we are aware, this is the first instance of a sliding window analysis of asymmetry conducted for any Cerithioidea species, leaving it uncertain whether this is a species-specific characteristic or a more widespread phenomenon within the group. Therefore, further research involving other Cerithioidea species is essential to explore the relationship between strand asymmetry, gene orientation, and its potential link to replication direction.

The sizes of the PCGs in *A. chloroticum* are consistent with those previously documented for the Cerithioidea, exhibiting conserved values for the genes *cox1*, *cox3*, *nad3*, *nad4*, and *nad4l*. In contrast, the genes *cox2*, *cob*, *nad1*, *nad2*, *nad5*, *nad6*, *atp6*, and *atp8* display variations in length among the species within the superfamily. For this second group, the sizes recorded in *A. chloroticum* corresponded with some of those previously reported, except for the *atp6*, which exhibited a length of 699 bp, representing the first record of this size. Additionally, all PCGs of *A. chloroticum* featured ATG start codons and TAA or TAG stop codons, which are widely used within the superfamily (Hilgers et al. 2016; Kim and Lee 2018; Ling et al. 2022; Xu et al. 2024). Furthermore, and

consistent with observations in other Cerithioidea species (Lee et al. 2019b; Xu et al. 2024), the RSCU analysis based on 3,741 amino acids, which estimates the deviation of synonymous codon usage from an even distribution, revealed that all codons were utilized. Notably, the most frequently used codons among the 13 PCGs were those with a high A+T content, e.g., TTA (Leu_2) = 7.56%, TTT (*Phe*) = 7.22%, ATT (*Ile*) = 6.55%. In contrast, codons with a high G+C content were rare, e.g., GCG (*Ala*) = 0.16%, TCG (Ser_2) = 0.16%, ACG (*Thr*) = 0.11%, CGC (*Arg*) = 0.08%. Among the preferred codons (Suppl. material 5, RSCU values > 1 indicating codon-usage preference), 30 codons ended with A (15/30) or T (15/30), illustrating that high-frequency codons in the PCGs of *A. chloroticum* tend to end in A or T. This pattern aligns with previous RSCU analyses in other Cerithioidea species, which also indicate a bias toward A/T at the codon-ending positions (Lee et al. 2019b; Xu et al. 2024).

Among the 22 identified tRNAs, all fell within the previously characterized size range for Cerithioidea, with lengths varying between 61 and 86 bp (Suppl. material 1). To the best of our knowledge, the secondary structure models of tRNAs proposed in this study represent the first available for the family Hemisinidae and the third for the superfamily Cerithioidea, following the semisulcospirids *Semisulcospira gottschei* (Martens, 1886) and *Hua aristarchorum* (Heude, 1889) (Lee et al. 2019b; Xu et al. 2024). The tRNA models of *A. chloroticum* conformed to the typical cloverleaf structure characteristic of these genes (Boore et al. 2005), with the exception of $tRNA^{Ser1}$, which lacked the dihydrouridine (DHU) arm. The loss of DHU arms in $tRNA^{Ser}$ has been observed in other animal groups, including mollusks, and the absence of this arm in $tRNA^{Ser1}$ had already been reported for the two Cerithioidea species with available secondary structure models (Lee et al. 2019b; Xu et al. 2024).

The ribosomal RNA genes of *A. chloroticum*, *16S-rRNA* and *12S-rRNA*, have lengths of 1,357 bp and 948 bp, respectively, similar to those previously reported for Cerithioidea (Suppl. material 1). Additionally, secondary structure models for both genes were obtained, which, to our knowledge, are the first complete models available for Hemisinidae. These structures displayed the typical domains, I–VI for *16S-rRNA* and I–IV for *12S-rRNA*. In general, when compared to previously available secondary structure models for mollusks (e.g., Lydeard et al. 2000; Guzmán et al. 2021; Mendivil et al. 2023), most of the stems were found to be conserved, particularly the long-range stems, with loops being the most variable regions. In the large subunit, we found the formation of a short-range stem (4 bp) within domain II, which had not been previously documented in other mollusk models, such as those available on the Comparative RNA Web-2 site (https://crw2-comparative-rna-web.org/). Regarding the small subunit, domains III and IV were found to be conserved, sharing stem and loop structures with the reference models (e.g., Hickson et al. 1996; Guzmán et al. 2021; Mendivil et al. 2023). Specifically, domain III exhibited minimal variation compared to the structure proposed by Vogler et al. (2016), based on a partial sequence of the *12S-rRNA* of *A. chloroticum*. The sequences of *A. chloroticum* published by these authors, from the localities of Candelaria (Misiones, Argentina) and Encarnación (Itapúa, Paraguay), showed 100% identity, representing a single haplotype. In comparison, the sequence obtained in this study revealed eight polymorphic sites relative to that previous one, although these variations did not impact the secondary structure.

In relation to mitochondrial genome architecture, it is worth noting that, in contrast to other animals where mitochondrial gene arrangements tend to remain relatively stable, extensive gene order rearrangements have been documented across every major lineage within Mollusca (Osca et al. 2015; Ghiselli et al. 2021). Within the context of the superfamily Cerithioidea, two rearrangements have been documented, altering the positions of $tRNA^{Gln}$ and $tRNA^{Arg}$. This has led to the recognition of two groups within Cerithioidea based on gene order: one that includes Turritellidae, Pachychilidae, and Batillariidae (order a in Fig. 4), and another that contains Pleuroceridae, Semisulcospiridae, Potamididae, Paludomidae, and Thiaridae (Kato et al. 2022), where *A. chloroticum* and the family Hemisinidae are now incorporated (order b in Fig. 4).

To elucidate the evolutionary affinities of members of the superfamily Cerithioidea, phylogenetic reconstructions have been performed based on both morphological and molecular data (e.g., Simone 2001; Lydeard et al. 2002; Strong et al. 2011). In this study, mitogenomic data were used to reconstruct phylogenetic trees through ML and BI approaches, based on two nucleotide datasets (PCG123, PCG12), which yielded identical topologies. However, they differed from those generated from the amino acid dataset in the affinities of Potamididae: (i) appearing as the sister group to Paludomidae + (Hemisinidae + Thiaridae) in the nucleotide datasets or (ii) as the sister group to Pleuroceridae + Semisulcospiridae in the amino acid dataset. Despite these differences, all obtained trees confirmed the monophyly of Cerithioidea with maximum support values. In all cases, the family Hemisinidae, represented by *A. chloroticum*, was shown to have a sister group relationship with Thiaridae, while Paludomidae grouped externally to both families: Paludomidae + (Hemisinidae + Thiaridae).

For Cerithioidea, both topologies have been previously obtained based on complete mitogenomes, with the one linking Potamididae as the sister group of Paludomidae + (Hemisinidae + Thiaridae) aligning with reconstructions by Ling et al. (2022), Yang and Deng (2022), and Yin et al. (2022), but differing from those obtained by Kato et al. (2022) and Xu et al. (2024), where Potamididae was shown to be more closely related to Semisulcospiridae + Pleuroceridae. Additionally, both topologies show that within Semisulcospiridae, *Semisulcospira libertina* (Gould, 1859) (NC_023364; Zeng et al. 2015) appears among *Koreoleptoxis* species. Based on geographic distribution and comparisons with GenBank, Xu et al.

(2024) proposed that this represents a misidentification, suggesting that the sequence may correspond to the semisulcospirids *Koreoleptoxis praenotata* (Gredler, 1884) or *K. davidi* (Brot, 1875). Although the differences between both topologies might be influenced by the still limited taxon sampling in mitogenome-based phylogenetic reconstructions, differences between the relationships recovered from analyses using different datasets (nucleotide-based vs. amino acid-based) have usually been found to be influenced by nucleotide composition affecting the amino acid composition, as well as differences in the patterns of strand asymmetry, which significantly influence the amino acid composition of the encoded proteins (Foster et al. 1997; Masta et al. 2009; Bernt et al. 2013b; Sun et al. 2018). While evaluating the relationship and impact of compositional biases at the DNA and protein levels on phylogenetic reconstructions of Cerithioidea is beyond the scope of this study, further research is essential to determine whether DNA bias can confound phylogenetic reconstruction based on amino acid sequences.

Moreover, the positioning of Potamididae as more closely related to the group comprising Paludomidae, Hemisinidae, and Thiaridae aligns with earlier phylogenetic reconstructions by Strong et al. (2011). This is consistent with our topologies derived the combined analysis of *16S-rRNA* and *28S-rRNA* markers, where the overall structure of our trees was congruent with some of the arrangements presented in the topologies obtained by Strong et al. (2011). In our trees, most of the currently recognized families within Cerithioidea exhibited high support values, with the exception of Batillariidae, where the Neotropical batillarids clustered more closely with Planaxidae, as previously noted by Ozawa et al. (2009). For this dataset, *A. chloroticum* formed a well-supported group together with *H. cubanianus* from Cuba in both phylogenetic analyses, supporting the monophyly of the family Hemisinidae, which was positioned as the sister group to Thiaridae + Paludomidae. This aligns with the findings of Neiber and Glaubrecht (2019), who suggest that the distribution and phylogenetic position of a clade comprising Thiaridae, Hemisinidae, and Paludomidae are consistent with a Gondwanan origin for this group.

These findings are particularly significant, as this is the first time the relationships of a member of the genus *Aylacostoma* have been explored within the phylogenetic context of Cerithioidea based on molecular data. In line with the results of this study, Simone (2001), using morphological data and including three species of *Aylacostoma* from Brazil, had previously identified a sister group relationship between *Aylacostoma* spp. and *M. tuberculata* (Thiaridae). However, given that there are two alternative hypotheses for the evolutionary affinities of Hemisinidae—the first being Paludomidae + (Hemisinidae + Thiaridae) based on mitogenomes, and the second, Hemisinidae + (Thiaridae + Paludomidae) based on single-gene markers—further studies including a larger number of taxa from the three families are needed to confirm the evolutionary affinities of Hemisinidae.

Conclusion

We successfully characterized the mitochondrial genome of the threatened South American gastropod *Aylacostoma chloroticum*, the first available for the family Hemisinidae. This genome contained the typical 37 genes of Metazoa, including 13 PCGs, along with two rRNA genes and 22 tRNA genes, and presented structural, compositional characteristics, and a gene arrangement consistent with those of other members of the superfamily Cerithioidea. Our phylogenetic reconstructions confirmed that *Aylacostoma* does not belong to the family Thiaridae as historically proposed, but instead belongs to Hemisinidae, which was supported as a monophyletic group in our analyses. We also identified alternative hypotheses regarding the relationships among Hemisinidae, Paludomidae, and Thiaridae that need further confirmation. Thus, this study represents a foundational step towards understanding the organization and structure of mitochondrial genomes in Neotropical hemisinids, as well as their evolutionary affinities.

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Supplementary material 1

Structural features of mitochondrial genomes in 19 representatives of the Cerithioidea superfamily

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Link: https://doi.org/10.3897/zse.101.142841.suppl1

Supplementary material 2

List of species of Cerithioidea used in phylogenetic analyses based on mitogenomes

Authors: Emanuel Forestello, Ariel A. Beltramino, Juana G. Peso, Roberto E. Vogler
Data type: docx
Explanation note: The species names and families are presented according to MolluscaBase (2024).
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Supplementary material 3

Evolutionary models used in the phylogenetic reconstructions based on mitogenomes

Authors: Emanuel Forestello, Ariel A. Beltramino, Juana G. Peso, Roberto E. Vogler
Data type: docx
Explanation note: The positions within codons are indicated by numbers 1, 2, and 3.
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Link: https://doi.org/10.3897/zse.101.142841.suppl3

Supplementary material 4

List of Cerithioidea species and their corresponding accession numbers used in phylogenetic analyses based on *16S-rRNA* and *28S-rRNA* gene sequences

Authors: Emanuel Forestello, Ariel A. Beltramino, Juana G. Peso, Roberto E. Vogler
Data type: docx
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Supplementary material 5

Relative Synonymous Codon Usage (RSCU) of protein-coding genes in the mitogenome of *Aylacostoma chloroticum*

Authors: Emanuel Forestello, Ariel A. Beltramino, Juana G. Peso, Roberto E. Vogler

Data type: docx

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Supplementary material 6

Additional images

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Data type: docx

Explanation note: **fig. S1.** Putative secondary structure of the 22 tRNA genes of *Aylacostoma chloroticum*. Conventional Watson-Crick pairings are represented by lines, and non-canonical G-U pairings are depicted with a dot (•). **fig. S2.** Inferred secondary structure of the 5' end of the *16S-rRNA* gene of *Aylacostoma chloroticum*. The domains I, II, and III are depicted. Conventional Watson-Crick pairings are represented by lines, and non-canonical G-U pairings are indicated with a dot (•). A stem unique to *A. chloroticum* is highlighted in color. **fig. S3.** Inferred secondary structure of the 3' end of the *16S-rRNA* gene of *Aylacostoma chloroticum*. The domains IV, V, and VI are depicted. Conventional Watson-Crick pairings are represented by lines, and non-canonical G-U pairings are indicated with a dot (•). **fig. S4.** Predicted secondary structure of the *12S-rRNA* gene of *A. chloroticum*. The four typical domains (I to IV) are depicted. Conventional Watson-Crick pairings are represented by lines, and non-canonical G-U pairings are indicated with a dot (•).

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